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and sex but also for duration and stress of traveling and time of blood sampling. Both patients and controls were asked to stop all medication (especially analgesics) and were asked not to use alcohol for at least 48 h before testing. To avoid the possible confounding effect of diurnal and seasonal variation, venous blood samples were collected from patients and controls between 8:30 and 11:00 A.M. on the same day. Plasma cortisol concentrations were measured in both patients and controls.

**Immunophenotyping and apoptosis.** Venous blood (4 mL) was taken in an EDTA-containing tube for white blood cell and differential counts. Heparinized blood was drawn for lymphocyte immunophenotyping by flow cytometry with dual-color direct and indirect immunofluorescence [7, 12]. Monoclonal antibodies (MAbs) were used to identify the following cell subsets: CD4, CD8, CD19, CD8CD28, CD8CD56, CD8CD11b, CD8CD38, and CD8HLA-DR (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Becton Dickinson Immunocytometry Systems, San Jose, CA). Apoptosis experiments were done as described [13] and simultaneously with the other immunologic tests on 4 consecutive days in cultured cell samples from 9 patients with CFS and their matched controls. In brief, PBMC were isolated by ficoll-isopaque density centrifugation from heparinized blood and cultured in the absence and presence of CD3 MAbs. Percentages of apoptotic cells were determined by in situ nick translation as described [13]. Positive controls included in these experiments were samples from human immunodeficiency virus-positive patients.

**Cytokine production.** Blood samples for cytokine measurements were collected into 4-mL endotoxin-free EDTA tubes (Vacutainer; Becton Dickinson, Rutherford, NJ). The plasma was immediately processed to avoid ex vivo cytokine production and release. Cytokine production was measured using a whole blood culture system as described elsewhere [9]. Interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-1 receptor antagonist (IL-1RA), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were measured with an RIA as described [9]. IL-1 $\alpha$  was also measured with an ELISA (provided by J. J. Castracane, Endogen, Boston). Transforming growth factor- $\beta$  (TGF- $\beta$ ) was measured with a bioassay as described [6] and with an immunoassay (Quantikine; R & D Systems, Minneapolis). Active TGF- $\beta$  was measured in serum; then total TGF- $\beta$  was measured after addition of acetic acid and urea, which release latent TGF- $\beta$  from the latency-associated peptide. The sensitivities of the assays were as follows: IL-1 $\alpha$ , 5 pg/mL (RIA) and 13 pg/mL (ELISA); IL-1 $\beta$ , 20 pg/mL; IL-1RA, 50 pg/mL; TNF- $\alpha$ , 40 pg/mL; and TGF- $\beta$ , 200 pg/mL (bioassay), 300 pg/mL (total TGF- $\beta$ , immunoassay), and 125 pg/mL (active TGF- $\beta$ , immunoassay). In each assay, reference samples with known concentration of cytokines were tested concurrently. Patients and controls were tested simultaneously to correct for intertest variability.

**Statistical analysis.** Since cytokines and cell-markers are known to have a non-Gaussian distribution, we used the Wilcoxon signed rank test to evaluate differences among cytokine production and T cell subsets between patients and controls. We used Spearman's  $\rho$  to test for correlation between cytokine production, immunophenotyping analysis, and clinical symptoms such as fatigue and depression.

## Results

**Immunophenotyping.** Absolute lymphocyte counts were similar for patients with CFS ( $1685 \pm 626/\text{mL}$ ) and controls

( $1698 \pm 464/\text{mL}$ ). The total numbers of CD2 (total T), CD4 (helper/inducer), CD8 (suppressor/cytotoxic), and CD19 (total B) lymphocytes did not differ between CFS patients and controls. The proportion of CD8 cells expressing CD11b (suppressor T cell) was significantly decreased in patients with CFS compared with controls ( $P < .05$ ; figure 1). We did not find increased expression of activation markers CD38 and HLA-DR on CD8 cells, nor did we find a significantly altered expression of CD28 ( $P = .054$ ). There was reduced expression of CD56 on CD8 cells, but this was significant only after one-tailed testing ( $P < .05$ ). Taken together, these findings indicate expansion of the cytotoxic T lymphocyte population with concomitant decrease of the suppressor T lymphocyte population. Percentages of apoptotic cells after overnight culture with or without stimulation with CD3 MAb were not different in patient and control samples (data not shown).

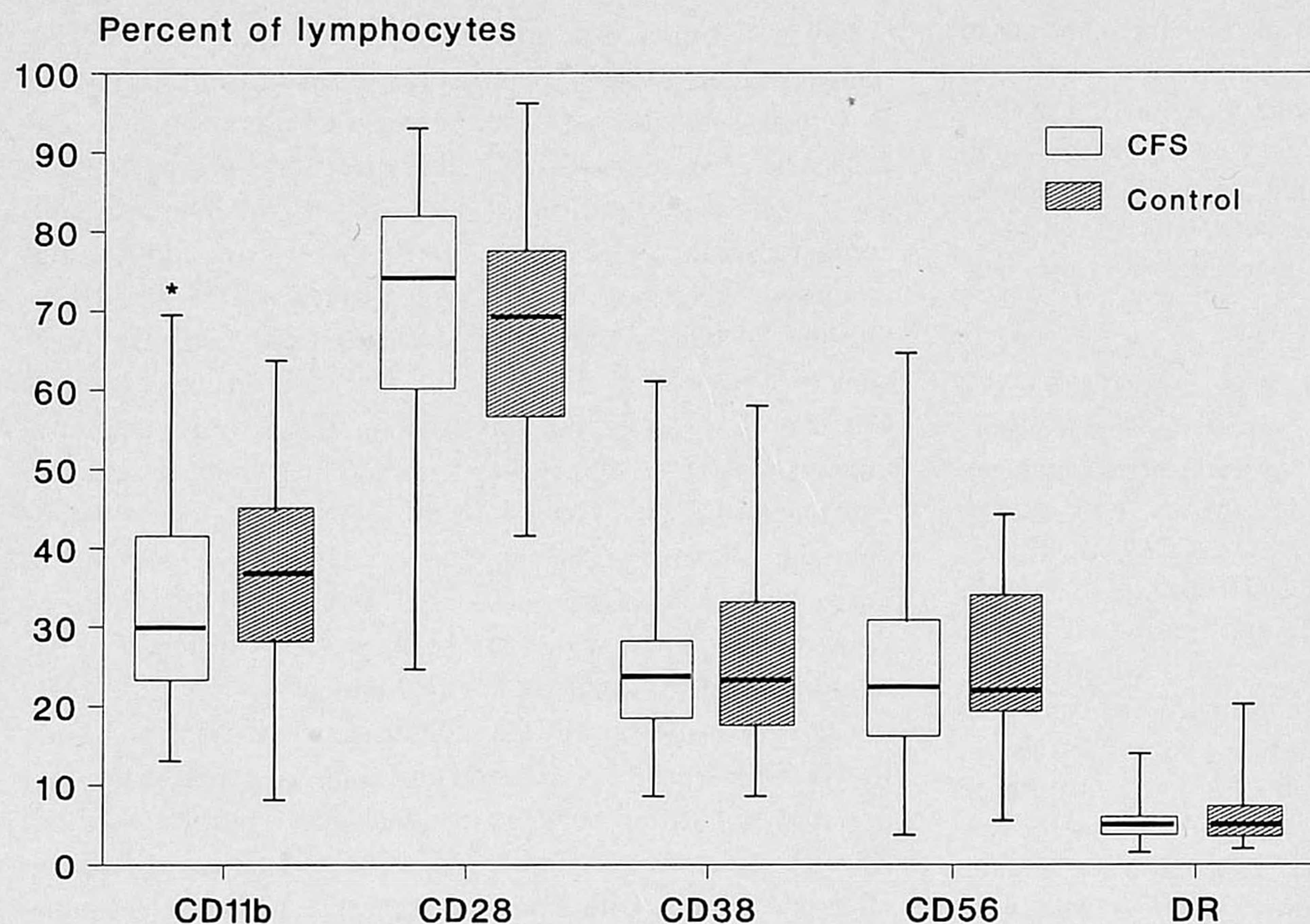
**Cytokine production.** Circulating concentrations of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , and IL-1RA and unstimulated cytokine production did not differ between patients and controls (data not shown). As shown in figure 2, TNF- $\alpha$  and IL-1 $\beta$  production after stimulation with lipopolysaccharide (LPS) was significantly lower in CFS patients than in their matched healthy controls ( $P < .01$  and  $.05$ , respectively). Although this difference was statistically significant, there was a large overlap between patients and controls. No differences were found in circulating total TGF- $\beta$  as measured by immunoassay ( $1.25 \pm 0.58$  vs.  $1.20 \pm 0.64$  ng/mL, patients vs. controls) or in TGF- $\beta$  as measured by bioassay. The concentration of active TGF- $\beta$  on immunoassay was below the detection limit for all patients and controls. Plasma cortisol concentrations were similar for patients and controls ( $0.36 \pm 0.22$  vs.  $0.30 \pm 0.14$  mg/mL, respectively).

Circulating cytokine concentrations correlated well with concentrations found in unstimulated whole blood cultures: IL-1 $\alpha$ ,  $r = 0.96$ ; IL-1 $\beta$ ,  $r = 0.69$ ; TNF- $\alpha$ ,  $r = 0.45$ ; IL-1RA,  $r = 0.74$  ( $P < .001$  for all). This is explained by the presence of these cytokines in plasma and the virtual absence of ex vivo cytokine production in the absence of a stimulus such as LPS. There was a low correlation between LPS-induced production of IL-1 $\beta$  and IL-1 $\alpha$  ( $r = 0.47$ ,  $P < .001$ ), IL-1 $\beta$  and TNF- $\alpha$  ( $r = 0.26$ ,  $P < .05$ ), and IL-1 $\beta$  and IL-1RA ( $r = 0.25$ ,  $P < .05$ ). No correlation was found between cytokine production and expression of activation markers. The average level of fatigue using the CIS rating scale (range, 1–7) was 5.8 (1.1 SD) for CFS patients and 1.8 (0.9 SD) for healthy controls ( $P < .001$ ). However, in patients with CFS, we found no correlation between CIS-subscale fatigue and LPS-stimulated production of cytokines.

## Discussion

The data presented here are consistent with those of some previous reports [4, 7, 14] but contrast with others [6, 8, 15]. This may be due to patient selection, choice of control

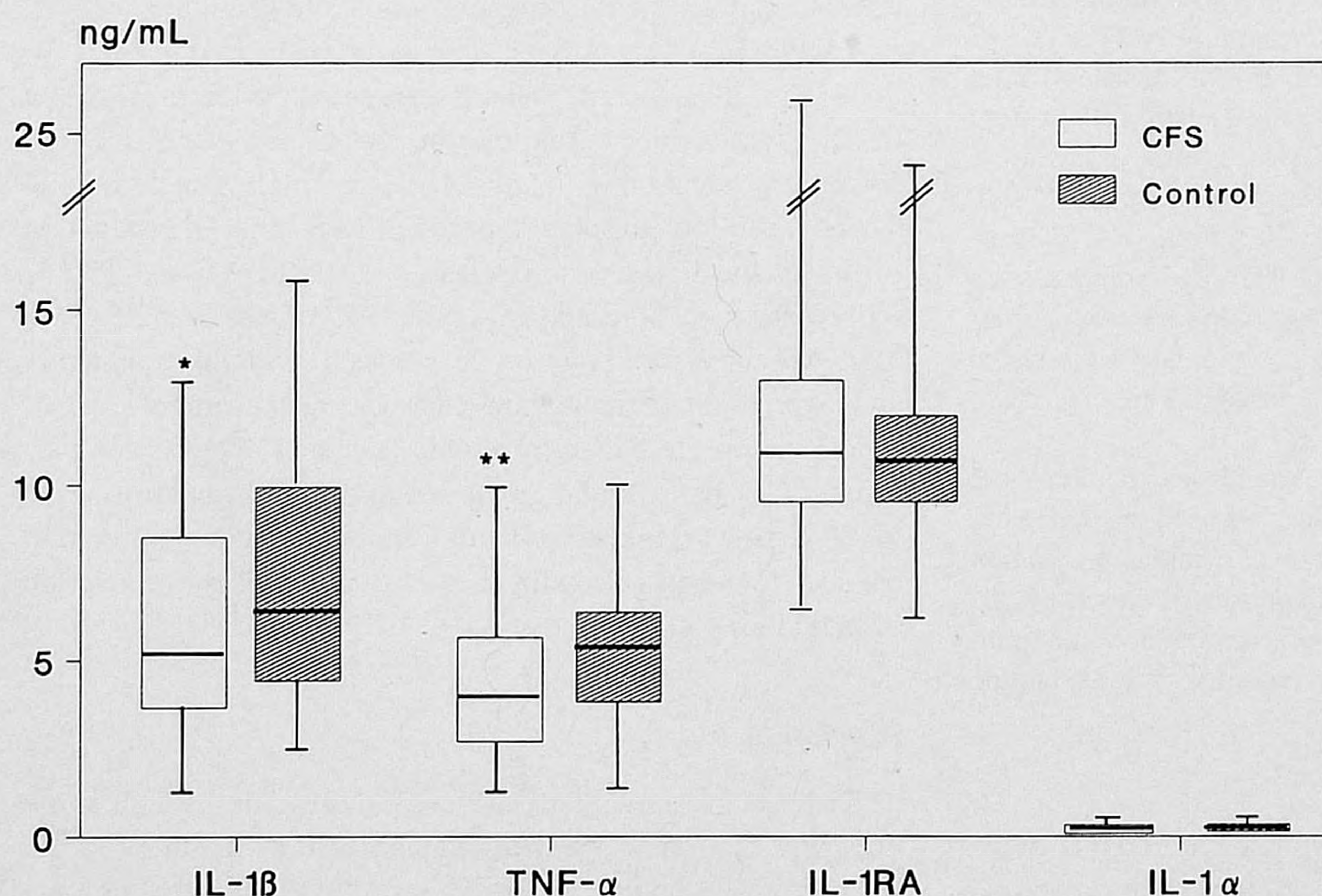




**Figure 1.** Expression of CD11b, CD28, CD38, CD56, and HLA-DR on CD8 cells. Boxes show 25th and 75th percentiles with median; whiskers indicate range. CFS = chronic fatigue syndrome. \*  $P < .05$ .

group, and differences in methods of assessment. We included self-referred patients in this study to avoid selection bias that is encountered when only patients referred to an infectious disease outpatient clinic are investigated. For the same reasons, we did not select by symptoms or previously detected laboratory abnormalities. For controls, we asked patients to bring a healthy nonfatigued neighborhood control, matched for sex and age. By doing this, we avoided

confounding effects such as duration and stress of traveling and corrected for unknown effects (e.g., environmental pollution). Furthermore, we used a whole blood culture system to measure cytokine production. This was done for practical convenience, but in addition, the method may reflect the in vivo situation more closely since manipulation, prestimulation, and possible selection of PBMC are minimized, and the role of plasma factors is included.



**Figure 2.** Cytokine production after lipopolysaccharide stimulation in whole blood culture at 24 h. Boxes show 25th and 75th percentiles with median; whiskers indicate range. IL = interleukin; TNF = tumor necrosis factor; RA = receptor antagonist. \*  $P < .05$ ; \*\*  $P < .01$ .



Previous studies have shown good correlation between IL-1 $\beta$ , IL-1RA, and TNF- $\alpha$  production in whole blood cultures and in isolated mononuclear cell cultures [16, 17]. In contrast to the results of Chao et al. [6], we found significantly decreased IL-1 $\beta$  and TNF- $\alpha$  production after LPS stimulation of peripheral blood cells. However, they separated the cells and did not add autologous plasma. Taken together, it can be hypothesized that plasma factors that inhibit cytokine production occur in CFS. Several inhibitory factors could be responsible for this. An increased concentration of TGF- $\beta$ , as found by Chao et al. [6], could explain the results, as TGF- $\beta$  is known to inhibit production of proinflammatory cytokines [6]. However, our finding that serum TGF- $\beta$  concentrations were similar in patients with CFS and concomitant controls does not support this view. As plasma cortisol measurements were normal, it seems unlikely that glucocorticosteroids inhibited the production of proinflammatory cytokines. Inhibitory cytokines, such as IL-4 and IL-10, or mediators, such as prostaglandins, could play a role. Further studies are underway to elucidate this issue. In contrast to others [8, 14, 15], we did not find increased concentrations of circulating IL-1 $\alpha$ .

We did find decreased expression of CD11b on CD8 cells, probably indicative of in vivo-activated CD8 T cells. This was also reported by Landay et al. [7] and Barker et al. [4]. We could not, however, detect significant differences in the expression of CD38 and HLA-DR, which have been reported to be elevated in patients with CFS [4, 7]. Since we did not find a correlation between high scores on the CIS fatigue questionnaire and CD8 cell subsets (CD11b, CD38, and HLA-DR), we doubt whether alterations in these subsets are indicative of having active CFS, as has been suggested [7]. We considered the possibility that in patients with CFS, a state of immune activation could lead to programmed cell death. In apoptosis experiments in 9 patients and their matched controls, there was no obvious difference in the percentages of cells dying due to programmed cell death (data not shown). Therefore, our data do not support the hypothesis that patients with CFS have a state of immune activation.

Our results show that subtle immunologic abnormalities can be detected in a randomly selected group of patients with CFS compared with a matched control group. However, since there is a wide range in the outcome of the immunologic tests used in this study, and because we did not find a significant correlation between symptoms and the outcome of these tests, the meaning of these findings is still unclear. It is clear that immunophenotyping of lymphocytes or assessment of cytokine production can be used neither to confirm nor to reject the diagnosis of CFS in an individual patient, nor to assess severity of illness.

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